IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

BOTTAZZI et al.

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Examiner: Jamroz

For: PHARMACEUTICAL COMPOSITIONS CONTAINING THE LONG PENTRAXIN PTX3

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Assistant Commissioner for Patents
Washington, DC 20231

Sir:

DECLARATION UNDER RULE 132

I, Alberto MANTOVANI, of Instituto di Ricerche Farmacologiche "Mario Negri", via Entrea 62, 20157 Milano, Italy, do hereby declare as follows:

1. I am a Professor of General Pathology at the School of Medicine, State University of Milan, Italy. A copy of my professional resume is attached.

2. I have reviewed the above-identified application and the claims.

3. I have been advised that an objection raised during the proceedings relating to the above is an alleged lack of expectation that one of ordinary skill would have been able to make and use the claimed invention, such as to treat diseases caused by bacteria, fungi, protozoa or viruses which show the capacity to bind to PTX3.

4. The following, which has been conducted by me or at my direction, is submitted as evidence that these concerns raised in the above are unfounded.

5. **PTX3 is capable to bind pathogens**

In Table 1 are reported some examples of pathogen agents which are above to bind PTX3.

TABLE 1

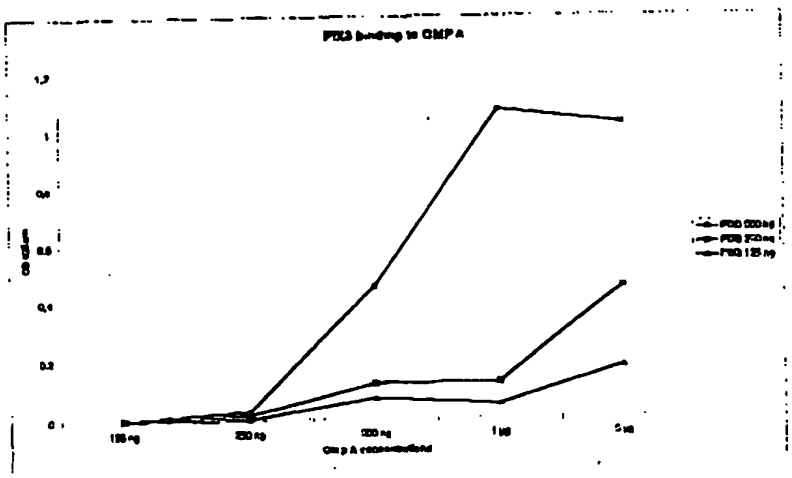
Pathogen	**Binding**
Aspergillus fumigatus	+
Pseudomonas Aeruginosa	+
Salmonella Tiphymurium	+
Staphylococcus Aureus	+

Legend: The indicated pathogens were incubated with a biotin labeled PTX3 (20 µg/ml) and analyzed b FACS with Streptavid-FITC. (+) indicate that PTX3 treated cells showed a significant increase of the mean fluorescence intensity (MFI) with respect to PTX3 untreated cells.

6. **PTX3 is capable of binding all Gram positive bacteria**

Figure 1 shows that **PTX3** is able to **bind** in vitro the outer membrane protein A (Omp A) which is expressed on the membrane surface of **all** the **Gram positive** bacteria.

Figure 1

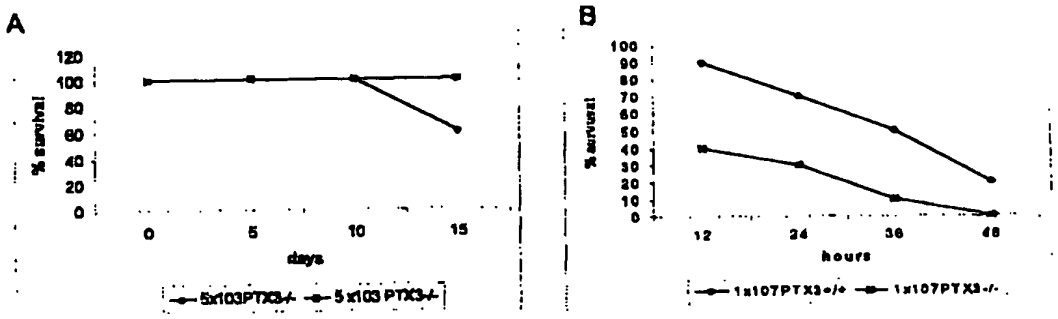


Legend: The indicated concentrations of Omp A have been absorbed on 96 multiwell plate.
Biotin labeled PTX3 at the indicated concentrations has been incubated on Omp A coated plate.
Streptavidin-HRP has been used to measure the relative amount of PTX3 bound to Omp A.

7. **Role of PTX 3 in Salmonella typhimurium and Pseudomonas aeruginosa infection**

To assess the role of PTX3 in protecting from Salmonella typhimurium and Pseudomonas aeruginosa infection, PTX3 -/- mice has been injected with the above mentioned bacteria and analyzed for survival in comparison with PTX3 +/+ mice.

Figure 2



Legend: A) The indicated doses of Salmonella Typhimurim were injected intraperitoneally in both PTX3^{+}/$^{+}$ and PTX3^{-}/$^{-}$ mice (n=8). Mortality was daily monitored. B) The indicated doses of Pseudomonas Aeruginosa were injected i.t. (intratracheally) in both PTX3^{+}/$^{+}$ and PTX3^{-}/$^{-}$ mice (n=8). Mortality was monitored every 12 hours.

The results reported in Figure 2 show that PTX3^{-}/$^{-}$ mice are more susceptible to Salmonella typhimurium and Pseudomonas aeruginosa infection then PTX3^{+}/$^{+}$ mice in terms of MST and mortality. This is a clear demonstration that PTX3 interaction with bacteria is required to protect against pathogens.

8. **<u>Role of PTX 3 in resistance to invasive pulmonary aspergillosis</u>**

PTX3 bound *Aspergillus fumigatus* conidia *in vitro*, this suggests a protective role for PTX3 in a murine model of invasive pulmonary aspergillosis.

PTX3 $^{-}$/$^{-}$ and $^{+}$/$^{+}$ mice were challenged with 2 x 10^{8} spores of *A. fumigatus* intratracheally. Mice were monitored for mortality, fungal load and pathology in the organs. As shown in Table 2, wild type mice survive to *A. fumigatus* in this model of infection. In contrast, in two different experiments performed, PTX 3 $^{-}$/$^{-}$ mice showed a MST of 3 days and a survival rate of 0%. *A. fumigatus* invasiveness was also assessed as fungal burden in lungs and brain. As shown in Table 2 the increased susceptibility of PTX3^{-}/$^{-}$ mice correlated with a dramatic increase in lung colonization at day three of

infection, with a 1000-fold increase in lung CFU in PTX3 $^{-/-}$ mice. The brain was not colonized in wild type mice, while in PTX 3$^{-/-}$ mice fungal burden in the brain was high (10^5 - 2 x 10^5 CFU/brain).

Mortality rate, MST and fungal burden in PTX3 $^{-/-}$ were equivalent to or worse than those obtained in PTX3 $^{+/+}$ mice after depletion of polymorphonuclear cells by treatment with anti-Gr-1 (RB6-8C5) (Table 2).

In two *in vivo* experiments PTX3$^{-/-}$ mice were treated with 20 µg of purified hPTX3 intratracheally at the time of challenge (day 0) and intravenously (day 1 and 2). As shown in Table 2 the phenotype was reverted and treated PTX3$^{-/-}$ mice behaved as PTX3$^{+/+}$ mice: mortality rate was reverted to 0/4 and MST was more than 60 days as in PTX $^{+/+}$ mice. Lung burden was reduced 4-fold by treatment. **The restoration of r sistance to invasive pulmonary aspergillosis (IPA) in PTX3 $^{-/-}$ mice by PTX3 administration confirms the critical and specific role of PTX3 in this fungal infection.**

TABLE. 2- Susceptibility of PTX3 -/- mice to invasiv pulmonary *aspergillosis*

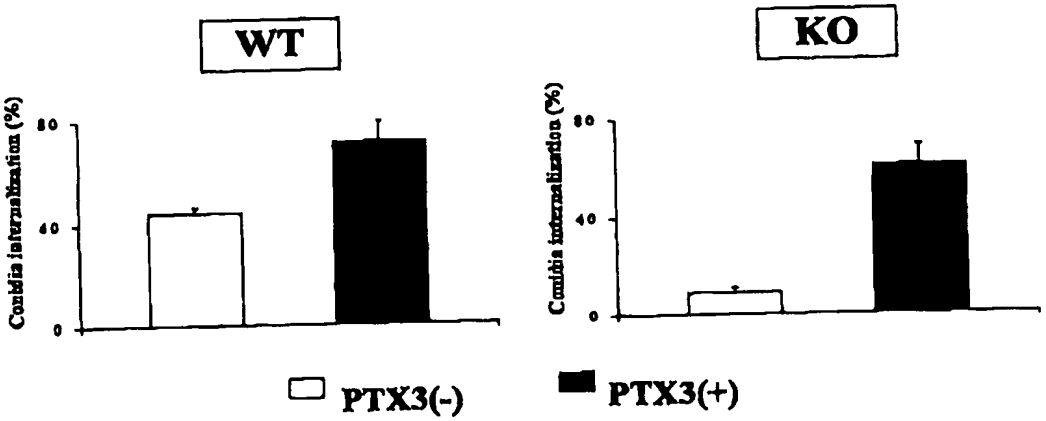
Mice	Treatment (a)	MST (days) (b)	Dead/total	Brain CFU (c)	Lung CFU (c)
Exp. 1					
PTX3 +/+	None	>60	0/3	0	8100
PTX3 +/+	RB6-8C5	4	4/4	34800	170100
PTX3 -/-	None	3	3/3	142200	706500
PTX3 -/-	RB6-8C5	3	3/3	187200	603750
Exp. 2					
PTX3 +/+	None	>60	0/6	ND	12900
PTX3 -/-	None	3	7/7	ND	233250
PTX3 -/-	PTX3*	>60	0/4	ND	60900

Legend: Mice were infected intratracheally with *A fumigatus* conidia (2 x 10^8/mouse) on day 0. (a) Mice were treated with RB6-8C5 monoclonal antibody (100 µg/mouse) intraperitoneally 2 h before fungal challenge to obtain PMN depletion or with(*) 20 µg PTX3 intratracheally on day 0 and intravenously on day 1 and 2. (b) MST: median survival time. (c) CFU were determined at day 3 after infection.

9. **PTX3 improve Phagocytosis of A. fumigatus conidia by alveolar macrophages an in vitro internalization assay.**

The ability of alveolar macrophases to ingest resting conidia in vitro, was significantly impaired in PTX3 $^{-/-}$ mice, as compared to PTX3 $^{+/+}$ mice (Fig. 3). However, PTX3 restored the phagocytic activities of cells from PTX3 $^{-/-}$ mice and potentiated PTX3 $^{+/+}$ mice (Fig. 3) This phagocytosis assay on PTX$^{+/+}$ macrophases is a further indication of the therapeutic activity of PTX3 in pulmonary infections.

Figur 3



Legend: Alveolar macrophases isolated from the indicated mice (2 x 10^5 cells/200 µl) obtained by plastic adherence from the bronchoalveolar lavage fluid, were incubated at 37° C for 2h with 10^6 conidia in 6 ml polypropylene tubes (N. 2063, Falcon), in 200 µl of Iscove medium containing 5 µg/ml polymixin B (Sigma) and 50 µl/ml gentamycin but no FCS to avoid non specific activation by serum components. Phagocytic cells were separated from non phagocytosed *A. fumigatus* cells by centrifugation on a fetal serum gradient. Harvest phagocytic cells was used for cytospin preparation. After Diff Quik staining fungal cell internalization was express according to the following formula: Conidia internalization = number of cell containing one or more fungal cells / 100 cells: In PTX3 (+), 20 µg/ml PTX 3 was added.

10. **Therapeutic function of PTX3 in a murine T-cell depleted model of invasive pulmonary asperigillosis**

Asperigillus fumigatus is a major opportunistic pathogen in immunodeficient patients and poses a formidable therapeutic challenge. It has been investigated whether administration of PTX3 was active in an invasive pulmonary aspergillosis model of allogeneic, T-cell depleted, bone marrow transplantation (BMT) in $PTX3^{+/+}$ mice. As shown in Table 3, combined systemic and local PTX3 administration caused a significant two-fold increase in survival time with two out of eight mice being cured.

Moreover, the lung CFU counts were drastically reduced (> four-fold) in PTX3-treated mice.

Table 3

Mice	Treatment	MST (days)	Dead/total	Brain CFU	Lung CFU
BMT	None	3	8/8	ND	814310
BMT	PTX3*	8^{+}	6/8	ND	187300^{+}

Legend: Mice underwent allogeneic T-cell-depleted BMT as described in Mencacci, A. et al. *Blood* **97**, 1483-90. (2001) were infected intratracheally (i.t.) with *A fumigatus* conidia ($2x10^8$/mouse) 7 days later. PTX3 was given on day 0 i.t. and on day 1 and 2 i.v. (*, 20 μg/mouse). $^{+}p<0.05$ compared to control mice (Mann Whitney U test).

As above mentioned:

1) PTX3 binds selected microbial agents, comprising conidia of *Asperigillus fumigatus, Pseudomonas aeruginosa, Salmonella tiphimurium, Staphylococcus aureus,* and all *Gram positive* bacteria;

2) PTX3$^{-/-}$ mice show higher mortality and reduction of the medial survival time when infected with pathogens;

3) PTX$^{-/-}$ mice infected with pathogens mentioned above show lower mortality and higher medial survival time when treated with PTX3;

4) susceptibility to *A fumigatus* infection of $^{-/-}$ mice was associated with defective recognition of conidia by alveolar macrophages and indicate that conidia opsonization by PTX3 direct binding is required to reverse defective phagocytosis; and

5) pulmonary aspergillosis infection, in a mouse model of allogeneic, T cells depleted, bone marrow transplantation can be prevented by PTX treatment thus indicating the therapeutic role of PTX3 also in $PTX3^{+}/^{+}$ immune compromised mice.
While the applicants do not believe it necessary to indicate or explain a mechanism of action, and without wishing to be bound by any such action, the applicants believe that the data presented indicate that PTX3 works as soluble pattern recognition receptor (PRR) and is useful as protective agent in pathogens infection. In particular, PTX3 appears to be effective when bound to the pathogen agent.
This mechanism of action can be extended not only to all fungi or bacteria which are capable of binding PTX3, but also to protozoa or viruses that show the same capability to bind PTX3.

11. Accordingly, in view of the above, I believe one or ordinary skill should appreciate that the claimed invention is supported by a disclosure which teaches one of ordinary skill how to make and use the invention of the claims.

12. It is also my understanding that an objection has been raised that anti-tumor activity by cloning human PTX3 into a murine mastocytoma p815 cell line would not be expected by one of ordinary skill in the art to expect successful treatment of tumors as claimed.

13. On page 9 to page 10 line 3 of the text of the present application however, are reported data about the anticancer activity of the compound according to the present

invention: "**Anticanc r activity:** a line of murine mastocytoma **P815** was transfected by electroporation with the expression vector pSG5 containing the cDNA of human PTX3 or its antisense.

Male DBA/2N CrlBR mice aged 8-10 weeks were subcutaneously injected with $1x10^5$ cells of P815 PTX3-producing clones or with clones containing the antisense gene. **Th mice were monitored 3 times daily for occurrence of tumours and once daily for survival.**

The results obtained are reported in Table 2 and show the efficacy of PTX3, in this experimental model of gene therapy, in bringing about healing of the animals and complete rejection of the tumour after the take of the inoculated tumour cells.

These results are statistically significant with p<9,91 (Fisher test) both as compared to controls and to the group treated with the antisense" (Emphasis added).

On page 11, Table 2 of the text are reported the data of the antitumoral activity of the compound according to the present invention.

"**TABLE 2**	IN VIVO ANTICANCER ACTIVITY OF PTX3
Clone[1]	Reject[2]
Parent **P815** (control)	4/25
P815-AS1 (antisense)	3/8
P815-PTX3-1 (sense)	**14/14***

1: 1 x 10^5 cells of the clone indicated were injected subcutaneously.
2: Number of animals that definitely reject the tumour out of total number of animals in which it took.

*** : p<0.01 as compared both to mice tr ated with par nt c lls and to mic tr ated with cells of th antis ns clones (Fisher t st)"** (Emphasis added).

14. In view of these aspects of the disclosure, as well as the entirety of the disclosure, I believe one of ordinary skill in the art should be convinced that the above-identified specification demonstrates the antitumor activity of PTX3.

15. To further characterize the antitumor activity of PTX3 however, the murine melanoma cell line B16 was stably transfected with the palsmid vector pSG5hPTX3 encoding for human PTX3, by me or at my direction.

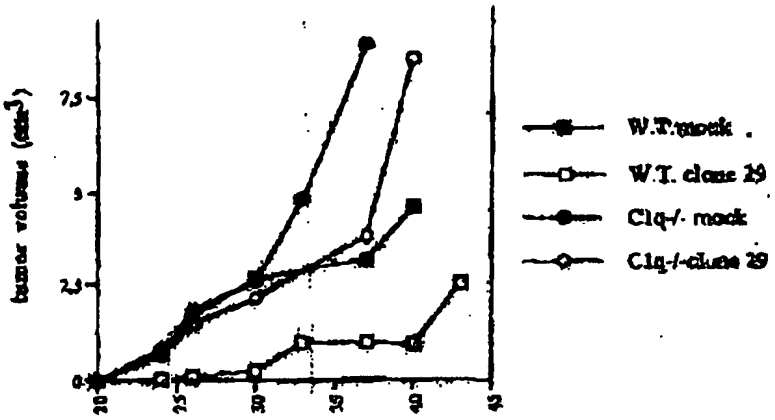
The B16 cell clone expressing the hPTX3 (PTX 29) was injected subcutaneously either in C57 mice or in C1qKO.

Untransfected B16 cells were used as control in both these mouse strains.

In the C57 mice, PTX3 transfected B16 cells showed **a significant** delay in tumor growth rate compared to untransfected parental cells (see Figure 4)

The observed delay of tumor growth rate of PTX transfected B16 cells was C1q dependant as C1q KO mice developed PTX 3 transfected and parental tumor at the same extent (see Figure 4).

Figure 4



Legend:
W.T. mock : C57/b6 mice treated subcutaneously with $1x10^5$ B16 cells;
W.T. clone 29: C57/b6 mice treated subcutaneously with $1x10^5$ B16 cells transfected with pSC5hPTX3;
C1q $^{-}\!/$ mock: C1q KO mice treated subcutaneously with $1x10^5$ B16 cells;
C1q $^{-}\!/$ clone 29: C1q KO mice treated subcutaneously with $1x10^5$ B16 cells transfected with pSG5hPTX3.

16. I believe one of ordinary skill in the art will appreciate from this evidence that the following main features characterize PTX3:

1) PTX3 is able to form a decamer by the establishment of disulfides bounds among its monomers,

2) The decamer of PTX3 is able to bind the first element of the complement classical pathway C1q (Bottazzi et al. 1997).

The experiment shown in Figure 4 highlight the antitumor activity of PTX3 even in the context of the B16 melanoma cell line and indicate that decamerization and C1q binding capacity of PTX3 is required for its antitumor activity.

17. The data reported in the application as filed and these above further presented data are a clear demonstration and confirmation of the antitumoral activity of the compound according to the invention.

18. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Further, declarant sayeth not.

Signed _____ Date 26/2/03

Alberto MANTOVANI